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Platelet Activation and Thrombosis: Studies in a Patient With Essential Thrombocythemia

P. Nurden, C. Bihour, M. Smith, J.M. Raymond, and A.T. Nurden

Service de Gastro-Entérologie, Unité de Soins Normalisés (USN) (J.M.R.) and URA 1464 CNRS (P.N., C.B., M.S., A.T.N.), Hôpital Cardiologique, Pessac, France

Recent advances permit the detection of activated platelets using specific monoclonal antibodies and flow cytometry. Nevertheless, there are few reports in which activated platelets have been studied over a period of time in patients at risk for thrombosis. Our patient S.D. has essential thrombocythemia and a prothrombotic state manifested in two major thrombotic episodes involving the portal vein and a mesenteric artery. Investigation revealed both spontaneous aggregation and hyperaggregability in response to ADP and the presence of activated platelets in platelet-rich plasma as revealed by flow cytometry. Interestingly, the activated platelets were recognized by an anti-RIBS ("receptor-induced binding site") monoclonal antibody that recognized bound fibrinogen but not by antibodies reactive with antigens whose presence on the platelet surface was secretion dependent. Treatment with aspirin inhibited spontaneous platelet aggregation but had little effect on the activated platelet profile. A change of therapy to ticlopidine suppressed expression of platelet activation markers. Treatment with ticlopidine has continued for 1 year so far without further thrombotic complications. © 1996 Wiley-Liss, Inc.

Key words: platelet activation, thrombosis, essential thrombocythemia, aspirin, ticlopidine

INTRODUCTON

Thrombotic and hemorrhagic events are recognized complications in patients with myeloproliferative disorders [1]. Essential thrombocythemia, one of the myeloproliferative states characterized by a persistent primary increase in platelet count, is a chronic disorder in which retrospective analysis has revealed thrombotic complications in 29%, hemorrhagic manifestations in 45%, and a combination of both in 14% of patients [2]. Effort has been concentrated on identifying risk factors for these events, focusing on such features as age, the full blood count, platelet aggregometry, plasma concentration of platelet secretory products, von Willebrand factor, and factors or byproducts of the coagulation cascade. However, such indicators have not proved useful for the prediction of thrombohemorrhagic complications in the individual patient [2,3]. In other research and clinical settings, flow cytometric analysis of platelet surface antigens has permitted the detection and quantification of levels of activated platelets in the circulation, the rationale for this approach being that increased levels represent a higher thrombotic risk [4–7]. However, there have been few long-term studies of patients and no evaluation of the effect of antithrombotic drugs on the observed activation. We present details of a patient with essential thrombocythemia with thrombotic complications in whom flow cytometry was used to demonstrate a state of in vivo platelet activation and to follow its evolution during therapy.

CASE REPORT

Patient S.D. is an adult woman who presented with a history of two major thrombotic episodes. The first, in October 1989, was a splenic and portal vein thrombosis. Subsequent imaging demonstrated persistent portal vein thrombosis, together with splenomegaly and ascites. Endoscopy showed the presence of esophageal varices, and endoscopic retrograde cholangiopancreatography outlined a normal hepatobiliary tree. The second major

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Address reprint requests to Dr. P. Nurden, Laboratoire d'Hémobiologie, URA 1464 CNRS, Hôpital Cardiologique, 33604 Pessac, France.

thrombotic episode occurred in August 1993, and was heralded by abdominal pain. At laparotomy, a small segment of nontorted, infarcted ileum was found, the histology of which showed ischemic necrosis. Subsequent intermittent abdominal discomfort has persisted. In addition to these major episodes, transient disturbances of vision and speech have been reported by the patient and may indicate a more generalized thrombotic tendency.

Specific hematological investigation is consistent with a myeloproliferative syndrome and essential thrombocythemia. Full blood counts since initial presentation have persistently documented a mild thrombocytosis, with platelet counts ranging from 450 to 654 $\times$ 10^9/liter, mean platelet volume always within the usual range, and other blood count indices otherwise normal. Morphological examination of the bone marrow at the time of the first clinically apparent thrombosis was normal. Repeat marrow aspirate and trephine in September 1993, revealed hypercellularity involving predominantly the megakaryocyte lineage, with dysplasia and abnormal clustering present. Hyperplasia of the granulocytic and to a lesser extent of the erythroid lineages was noted. In addition to the cytologically abnormal megakaryocytes, a small increase in reticulin staining was seen, reflecting slight myelofibrosis. It is believed that the splenomegaly (see above) is related to the splenic vein thrombosis rather than extramedullary hemopoiesis, a conclusion in part supported by the fact that a ^{59}Fe study performed in January 1994, did not show increased uptake in the spleen. Results of other tests for the presence of a procoagulant state, including antithrombin III, proteins C and S, euglobulin clot lysis time, plasminogen activator inhibitor (PAI) and tissue-type plasminogen activator (t-PA) levels, fibrinogen, and fibrin breakdown products (D-dimer assay) were all within normal limits. All studies were performed after informed consent was obtained. Control donors were adult laboratory staff.

MATERIALS AND METHODS

Flow Cytometry

Characteristics of control platelets activated by ADP in PRP. Venous blood samples were withdrawn by clean venipuncture from control donors directly into 3.8% (wt/vol) sodium citrate (1 vol anticoagulant to 9 vol blood). The initial 2 ml of blood was discarded. Platelet-rich plasma (PRP) was prepared by centrifugation at 120*g* for 10 min at room temperature. For in vitro platelet activation, volumes (10μl) of PRP were added to 100μl Hepes-buffered modified Tyrode's (HBMT) consisting of 137 mM NaCl, 2 mM KCl, 12 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 1 mM $MgCl_2$, 5.5 mM glucose, 5 mM Hepes, 0.1% (wt/vol) bovine serum albumin (BSA), pH 7.4 containing 2 mM $CaCl_2$. Preadded to the tube was one of the following monoclonal antibodies used as ascites fluid or isolated IgG; the antibodies were VH10 (IgG, 5μg/ml), recognizing P-selectin, a glycoprotein (GP) expressed on the platelet surface following secretion [8]; F26 (IgG, 5 μg/ml), specific for a receptor-induced binding site (RIBS) on platelet-bound fibrinogen [9]; and MOf11 (ascites fluid, 1/700 dilution), raised against a lysosomal membrane protein (CD 63) also expressed during platelet secretion. Bx-1 (IgG, 10 μg/ml), recognizing GP Ib, was used as a positive control [8]. The concentrations given were those shown in preliminary studies to be saturating for the glycoprotein being targeted. Also included in the tube was ADP (Sigma Chemical Co, St. Louis, MO) or, in some studies, TRAP-14-mer peptide (Neosystem, Strasbourg, France) at the concentrations described in the text. TRAP-14-mer peptide activates platelets through the seven transmembrane domain thrombin receptor [10]. Samples were not stirred after an initial mixing.

Presence of activated platelets in the PRP of the patient. The patient's platelets have been tested in our laboratory on four occasions: in November and December 1993, and in January and December 1994. It should be noted that these investigations began in the period following the second thrombotic event. Venous blood samples were drawn into 3.8% (wt/vol) sodium citrate, and PRP was prepared as described above. Aliquots (10μl) of PRP were added immediately after preparation to polystyrene tubes preloaded with 100 μl HBMT containing a predetermined saturating concentration of one of the above-mentioned monoclonal antibodies. Here, no platelet agonist was added. PRP from control donors was processed in parallel.

Flow cytometry. At this stage, stimulated control platelets in buffer and platelets from the patient were treated identically. After 15 min at room temperature, a predetermined saturating amount of second antibody, fluorescein (FITC)-conjugated affinity-purified $F(ab')_2$ fragments of sheep antibody to mouse IgG (1:40 dilution; Silenus Laboratories, Hawthorn, Australia), was added. After 15 min in the dark, samples were examined using a Becton-Dickinson FACScan flow cytometer (Becton-Dickinson Ltd., Le Pont de Claix, France) equipped with an argon-ion laser set at an excitation wavelength of 488 nm. Samples were first analyzed by forward- and right-angle light scatter, and the gate was set to include the majority of platelets. Fluorescence was detected in the 530 nm channel. Prior to passage, samples were diluted with 2 ml HBMT to ensure that the flow rate through the laser beam did not exceed 3,000 events per second. Fluorescence histograms were obtained for 10,000 cells, data being compiled and analyzed using the LYSYS II software (Becton-Dickinson). Histograms were composed from fluorescence data obtained using gain settings in the logarithmic mode. Antibody binding was expressed as the percentage of platelets positive for the antibody or as mean fluorescence intensity (MFI) expressed on a

linear scale. The gate for activated platelets was set to include <1% of the events seen when identical test samples were incubated without the murine monoclonal antibody. Nonspecific binding of mouse IgG was evaluated using an irrelevant mouse monoclonal antibody at concentrations identical to those of the test monoclonals.

Platelet Aggregation

Citrated PRP from patient S.D. was tested in a PAP-4 aggregometer (BIO/DATA Corporation, Horsham, PA) under constant stirring at 37°C. Samples were incubated with or without ADP (1–10 μM; Sigma). These analyses were performed in parallel with the detection of activated platelets by flow cytometry.

RESULTS

Binding of Activation-Dependent Monoclonal Antibodies to Control Platelets Activated In PRP

Initial experiments concerned the expression of activation-dependent markers on normal platelets stimulated with ADP in PRP. Typical results are shown in Figure 1. After incubation with 10 μM ADP, the bulk of the platelets bound F26, an anti-RIBS monoclonal antibody that recognizes an epitope expressed on fibrinogen after the protein has bound to the GP IIb-IIIa receptor. It should be noted that the labeled platelets were highly positive for this antibody, suggesting that a significant proportion of the complexes had bound fibrinogen. Taking into account interdonor variability in the platelet response to ADP, in vitro experiments similar to those depicted in Figure 1 showed that normal platelets began to react positively with F26 when stimulated with upwards of 0.1–0.5 μM ADP (data not shown). In the absence of agonist, few platelets were recognized by F26 (also see below). With 10 μM ADP, binding of VH10 or MOf11 was variable, showing that platelet activation under these conditions can be accompanied by a partial secretion of granule contents, a finding previously shown for α-granules and lysosomal granules by others [11,12]. With lower doses of ADP, few platelets underwent secretion. Most of the platelets were also recognized by F26 when stimulated with 50 μM TRAP-14-mer peptide (a dose that induces maximal secretion under our conditions). In contrast to ADP, TRAP-14-mer peptide induced a much more extensive binding of both VH10 and MOf11. These results therefore establish that, under our experimental conditions, we were capable of identifying platelets that had been activated both with and without secretion. Controls in which the selected monoclonal antibodies were replaced by an irrelevant mouse IgG were negative.

When GP Ib was analyzed, a decreased surface expression was seen for both ADP and TRAP-14-mer, although the decrease was greater with TRAP-14-mer (Fig. 1).

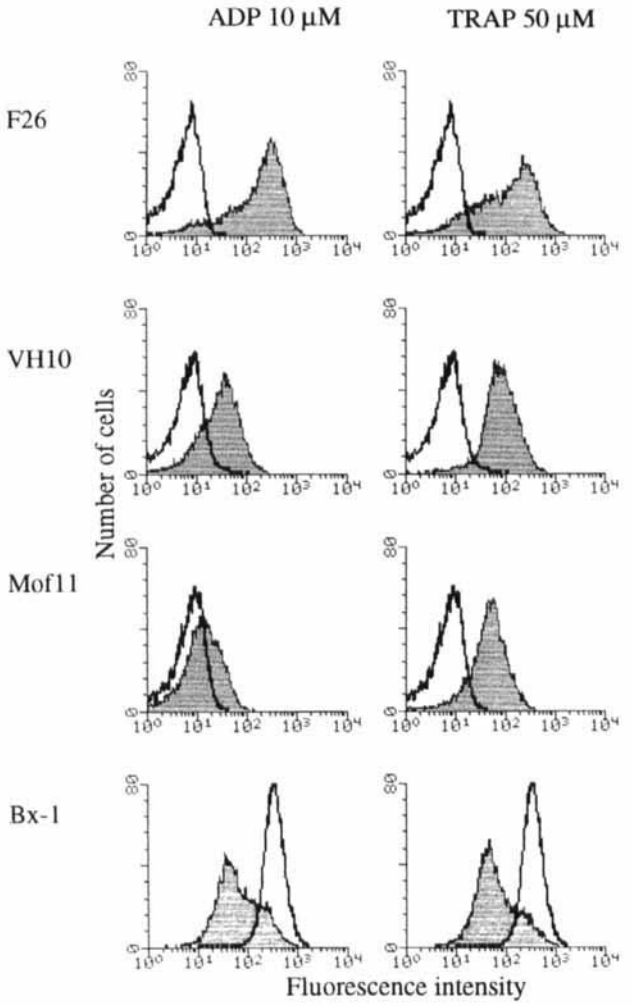

Fig. 1. Flow cytometric analysis of normal human platelets activated in vitro. Unstirred samples of citrated PRP were incubated with the monoclonal antibody in the presence or absence of 10 μM ADP or 50 μM TRAP-14-mer peptide. After 15 min at room temperature, FITC-labeled F(ab')$_2$ fragments of sheep antibody to mouse IgG were added, and the binding of each monoclonal antibody was evaluated by flow cytometry as described in Materials and Methods. Shaded areas show the analysis of platelets after stimulation with the agonist; open areas are control incubations performed in the absence of agonist. Gates were set to include <1% of the events seen when unstimulated platelets were incubated with identical amounts of irrelevant mouse monoclonal IgG.

These results confirm previous studies on the effect of activation on GP Ib distribution in platelets [8,13].

Studies on Patient S.D.

Our first analysis, performed in November 1993, consisted of evaluating the direct binding of activation-dependent monoclonal antibodies to platelets in PRP. Significantly, screening for platelet-bound fibrinogen with F26, a direct measure of GP IIb-IIIa complex activation, revealed a large subpopulation of activated platelets (Fig.

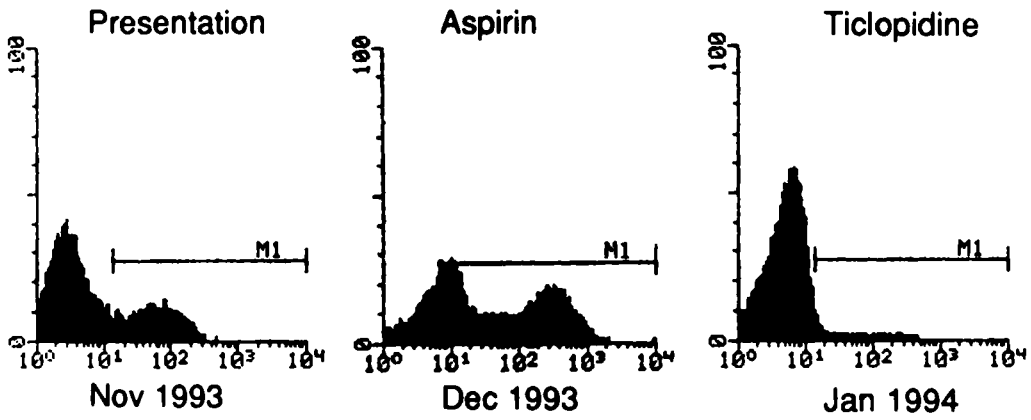

Fig. 2. Fluorescence histograms of patient S.D.'s platelet reactivity with the F26 monoclonal antibody, assessed on three separate occasions. Results from November and December 1993, at presentation and during aspirin treatment, respectively, show the presence of surface-bound fibrinogen on platelets in PRP, indicating GP IIb–IIIa activation. Analysis in January 1994, 1 month after the start of ticlopidine medication, shows that the bulk of the platelets are no longer expressing the activation antigen recognized by F26. M1 represents the gate containing <1% platelets when the patient's platelets were incubated with an irrelevant murine monoclonal antibody.

2). This was estimated to be 43% of the total platelet population. For a series of 15 normal subjects examined under identical conditions, the percentage of platelets recognized by F26 was 2.6% ± 3.0%. Thus, the level of activated platelets detected for patient S.D. was highly significant. Furthermore, close examination of the histograms showed that the subpopulation of the patient's platelets recognized by F26 had undergone a considerable shift in MFI, suggesting that the activation of GP IIb–IIIa was extensive. In contrast, few platelets expressing increased levels of P-selectin or CD63 and recognized by VH10 or MOf11 were seen (data not illustrated). For example, the MFI of the patient's platelets binding VH10 was 4.0 ± 1.7 arbitrary units of fluorescence, similar to that for normal controls. GP Ib expression (Bx-1) on the patient's platelets was within normal limits. As is illustrated in Figure 3, platelet aggregometry performed on the same PRP showed spontaneous aggregation, a response that was enhanced by low doses of ADP: 0.5 μM ADP produced 54% aggregation (maximal intensity); the parallel response of platelets from a series of controls with this dose of ADP was less than 10%.

The patient was placed on aspirin, 250 mg daily. Follow-up 1 month later showed only a partial resolution of the demonstrated abnormalities; spontaneous aggregation was no longer seen, with platelet aggregation curves with ADP as expected for a patient on aspirin (Fig. 3). It should be noted that the response to 0.5 μM ADP was rapid even if the second-phase aggregation did not occur. Significantly, activated platelets positive with the monoclonal antibody F26 in flow cytometry were again present in the PRP (Fig. 2). Results with VH10 and MOf11 remained negative. The persistence of the activated platelets prompted a trial change of antiplatelet medication to ticlopidine, 250 mg twice daily. Follow-up 1 month later showed that the number of activated platelets detected by F26 had now fallen dramatically (Fig. 2). Furthermore, with testing in the aggregometer, not only was spontaneous platelet aggregation not seen, but the platelets responded little to low-dose ADP (Fig. 3). Their response increased with high amounts of ADP, although it never attained the levels seen for normal platelets (not illustrated). The patient continues on a lower ticlopidine regimen (250 mg once daily), with a stable full blood count and no further clinically detectable thrombotic episodes to date. Her platelets were reexamined in December 1994, at which time activated platelets (F26) again were not detected (not illustrated).

DISCUSSION

This report concerns a patient with essential thrombocythemia and a recent history of arterial thrombosis. An association between myeloproliferative disorders and portal vein thrombosis has previously been described [14]. A distinguishing feature of our study is the fact that the presence of activated platelets in PRP was associated with spontaneous platelet aggregation. Furthermore, we were able to follow the changes that occurred during antiplatelet therapy. An important question relates to the mechanism giving rise to activated platelets in this patient. One hypothesis is that the abnormal megakaryocyte clone is producing platelets unusually susceptible to activation and sensitive to ticlopidine. Alternatively, the presence of the thrombotic mass in the portal vein, which after 5 years is almost completely organized, or other vessel lesions may be causing ongoing systemic platelet activation. In either situation, the flow cytometric results are

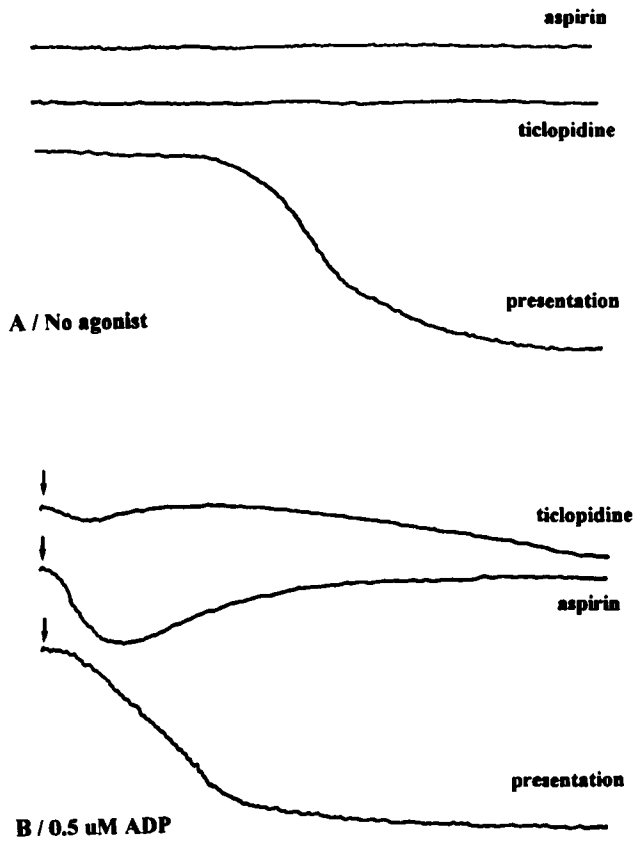

Fig. 3. Spontaneous aggregation seen in PRP from patient S.D. and the effect of antiplatelet therapy. Aliquots of citrated PRP were incubated with stirring at 37°C in the presence or absence of 0.5 μM ADP. Aggregation was tested using the same PRP preparations as were analyzed for the presence of activated platelets in Figure 2. A: Spontaneous aggregation tracings obtained at presentation (November 1993) and after aspirin (December 1993) or ticlopidine treatment (January 1994) are compared. B: Platelet response to 0.5 μM ADP (the vertical arrows mark the addition) from the same bleedings.

interesting, particularly with respect to the differences in the platelet activation profile seen during treatment first with aspirin and then with ticlopidine. Monoclonal antibodies detecting anti-RIBS epitopes on platelets are a relatively new development in the detection of activated platelets via flow cytometry [4]. A great advantage is that they detect platelets that have been stimulated with agonists such as ADP under conditions where the surface expression of secretion-dependent markers such as P-selectin is limited. PAC-1, an IgM murine antibody that binds directly to the RGD-binding domain of activated GP IIb–IIIa complexes [4] is an alternative probe, although it was not used in our study.

F26 recognizes fibrinogen that is bound to the GP IIb–IIIa complex [9]. The fibrinogen may originate from the plasma or may represent α-granule fibrinogen that becomes bound to GP IIb–IIIa complexes during, or shortly after, the induction of the release reaction. The fact that a subpopulation of the patient's platelets was highly positive for F26 but virtually negative for P-selectin and CD63, whereas platelets stimulated in vitro with a dose of TRAP-14-mer peptide capable of inducing extensive secretion were highly positive for F26, VH10, and MOf11, strongly suggests that thrombin was not the physiologic agonist responsible for the activation of the patient's platelets. It should be emphasized that our studies were all performed on platelets in PRP. This strategy enabled us to correlate the level of activated platelets with the platelet aggregation response under conditions of routine platelet function testing as performed in our laboratory. This choice means that we cannot rule out that some platelet activation was occurring during the preparation of the PRP. However, we have never seen high levels of F26-positive platelets in control PRP, showing that, at the very least, the patient's platelets were unusually sensitive to activation.

Much of what is interesting in this study lies in the fact that activated platelets were detected shortly after an arterial thrombosis had been diagnosed. It has long been assumed that the presence of activated platelets in the circulation suggests an increased thrombotic tendency. However, hard evidence to substantiate this assumption has not been forthcoming. A plausible explanation for this patient is that vascular lesions, possibly related to the portal vein thrombosis, are causing the activation that we detect. Interestingly, the spontaneous ex vivo platelet aggregation as initially seen was blocked by aspirin despite the persistent expression of activation-dependent markers on the platelet surface. Aspirin acts by inactivating cyclooxygenase and does not effect the primary platelet response to ADP, a finding confirmed in Figure 3. Such results imply that the observed platelet activation occurs independently of the formation of endoperoxides and thromboxanes, whose production is inhibited by aspirin. Thus aspirin was affecting one of the parameters of a prethrombotic state, spontaneous platelet aggregation, but not the binding of fibrinogen to GP IIb–IIIa complexes. These results led us to change the medication. With ticlopidine, activated platelets were no longer detected, and spontaneous aggregation was also no longer present. As was expected for this drug, a greatly decreased aggregation response of the patient's platelets to low doses of ADP was likewise present. Much evidence from ex vivo studies suggests that ticlopidine selectively inhibits the ADP-induced pathway of platelet aggregation [15]. There are a number of putative ADP receptors under consideration, and these include a receptor with high affinity for the ADP analogues 2-methylthio-ADP and 2-(p-azidophenyl)-ethylthio-ADP, which is linked into the adenylate cyclase pathway of platelet activation [16,17]. Recent studies have confirmed that ticlopidine-like drugs selectively affect the ability of ADP to induce fibrinogen

binding to GP IIb–IIIa through this receptor [18]. The implication, therefore, is that ADP is somehow involved in the platelet activation that we are detecting.

The current platelet surface marker profile of our patient suggests that the thrombotic risk is now significantly reduced. Nevertheless, we emphasize that our study applies to a single patient and that caution is needed in the interpretation of the findings. For example, it is possible that the thrombosed portal vein was itself responsible for the activation and that the activated platelets were secondary to an event that had already occurred. Nonetheless, we believe that our study suggests that a combination of platelet aggregometry and an analysis of activation-dependent surface markers may help in predicting the thrombotic risk in myeloproliferative disorders and that the application of this protocol or related protocols to other patients appears justified. Bellucci et al. [2] demonstrated the prolonged clinical course of essential thrombocythemia and suggested that a platelet count above $1{,}000 \times 10^9$/liter or a vascular risk or complication should favor chemotherapy over an antiaggregatory drug. With the help of modern flow cytometric techniques, it is now possible that the platelet activation state will also be of help, directing therapy when the most appropriate therapeutic option is uncertain.

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